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Oxygen- and Nitrogen-Dependent Sulfur Metabolism in the Thiotrophic Clam *Solemya reidi*

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Abstract. We investigated aerobic and anaerobic thiotrophic metabolism by the gutless clam *Solemya reidi* and its intracellular symbiotic bacteria. Mean environmental sulfide concentrations in porewater next to animals varied from a high of 888 μM to a low of 17 μM in different sediment samples, while mean thiosulfate concentrations were very low (1–13 μM). The blood of freshly collected clams contained up to 300 μM thiosulfate but little sulfide ($\leq$12 μM). In experimental incubations, clams were able to take up thiosulfate, yet under no conditions could the animals concentrate thiosulfate above external concentrations. Thiosulfate accumulation in the blood during incubations was the result of aerobic but not anaerobic sulfide oxidation by the animals. This finding and previous observations of the presence of high concentrations of thiosulfate in the blood of field-caught clams indicate that the animal portion of the symbiosis normally functions aerobically. The intact symbiosis exhibited nitrate and nitrite respiration under anoxic conditions. Nitrate respiration was stimulated by sulfide, as well as thiosulfate, while nitrite respiration was only stimulated by sulfide. Nitrate respiration also occurred when whole animals were under oxic conditions. Respiration measurements showed that the bacterial symbionts were capable of direct sulfide oxidation. Sulfide-stimulated oxygen consumption by bacterial preparations from the gills of mud-maintained clams reached a maximum rate at 25 μM sulfide and showed no apparent inhibition at sulfide concentrations up to 1 mM sulfide.

Introduction

Solemya reidi is a gutless, marine protobranch bivalve that lives in reduced sediments such as sewage outfall zones and pulp mill effluent sites (Reid, 1980; Reid and Bernard, 1980). The clam contains intracellular, chemolithotrophic bacterial symbionts within specialized cells in its gills (Felbeck, 1983). The bacteria use the energy from the oxidation of reduced sulfur compounds to fix and reduce CO_2 and subsequently translocate the fixed carbon to symbiont-free tissues of the animal, resulting in a net autotrophic existence of the symbiosis (Felbeck, 1983; Fisher and Childress, 1986; Anderson *et al.*, 1987). This autotrophic, sulfur-dependent mode of nutrition is called thiotrophy (Vetter, 1991).

Based on experiments done at high sulfide concentrations, Powell and Somero (1985) concluded that sulfide oxidation occurs in the animal tissue of *S. reidi* and not in the symbiotic bacteria. The authors identified intracellular, ferric iron-containing granules (originally called sulfide-oxidizing bodies, referred to as granules in this manuscript) and based upon a colorimetric assay suggested that granules were responsible for sulfide oxidation in *S. reidi* (Powell and Somero, 1985). The products of this oxidation were not identified. Subsequent studies revealed that isolated mitochondria of *S. reidi* are also capable of sulfide oxidation and that the mitochondria couple sulfide oxidation to aerobic respiration and ATP production (Powell and Somero, 1986; O'Brien and Vetter, 1990). The mitochondria oxidize sulfide exclusively to thiosulfate (O'Brien and Vetter, 1990). In addition, high concentrations of thiosulfate are found in the blood of field-caught animals and clams experimentally exposed to sulfide (Anderson *et al.*, 1987). These findings suggested that the mitochondria may also have a role in sulfide detoxification.

Current models propose that the detoxification of sulfide is an oxygen-dependent process that occurs only in the animal tissues of *S. reidi* (reviewed by Somero *et al.*, 1989). The oxidation results in the production of thio-

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sulfate. Thiosulfate is subsequently transported by the animal to the bacteriocyte where it is further oxidized by the bacteria, again by an oxygen-dependent respiratory process.

Aspects of this model describing thiotrophic metabolism are not proven or they are inaccurate because of a lack of direct experimental evidence or the technical difficulties in early experiments. First, there is little information on the environmental sulfide conditions in the clam's habitat. Although the bulk sulfide concentrations in sediment samples are highly variable, ranging from low micromolar concentrations up to 22 m*M* (Childress and Lowell, 1982; Vetter *et al.*, 1989), the concentration near the animals is not known. Data suggest that the optimal sulfide concentrations for the animals are 100 μM or below (Anderson *et al.*, 1987, 1990). In addition, the concentrations of other possible substrates (including thiosulfate, which is found in the blood of freshly caught clams) in the animals' burrows are not known.

Second, bacterial symbionts may be important agents of direct sulfide oxidation in the symbiosis in the presence of environmental sulfide concentrations. Recent experiments in our laboratory have shown that enriched bacterial suspensions show stimulation of $^{14}CO_2$ uptake in the presence of 500 μM sulfide and exhibit nitrate and nitrite respiration under anoxic conditions in the presence of 200 μM sulfide (Dr. Barbara Javor, pers. comm.). In addition, *in vivo* measurements of spectral changes of intracellular hemoglobins (Doeller *et al.*, 1988) and *in vitro* accumulations of elemental sulfur in isolated gill ctenidia exposed to oxygen and sulfide (Vetter, 1990) suggest that sulfide and oxygen enter the bacteriocyte directly from the seawater.

Third, the effect of anoxic conditions on sulfur metabolism of the host and symbionts needs additional study. Anderson *et al.* (1990) have shown that animal tissues of *S. reidi* maintain aerobic metabolism in the presence of sulfide concentrations up to 100 μM, but they switch to anaerobic metabolism (fermentation) in the presence of oxygen at higher sulfide concentrations. This switch is due to poisoning of cytochrome *c* oxidase and aerobic respiration (Anderson *et al.*, 1990). Whole animal experiments under conditions of low O_2 or at sulfide concentrations above 250 μM showed a loss of net autotrophy, suggesting that bacterial metabolism was inhibited (Anderson *et al.*, 1987). However, the anaerobic sulfide-oxidizing capacity of the symbionts was never measured and the clams were incubated in surface seawater that does not typically contain the alternate electron acceptor nitrate that some sulfur-oxidizing bacteria can use for anaerobic respiration. Thus, the absence of net autotrophy observed could be due to the absence of nitrate. Net CO_2 uptake might be enhanced at high sulfide concentrations or at low O_2 concentrations under conditions promoting anaerobic respiration in the bacteria.

High nitrate reductase activity has been observed in *S. reidi* (Felbeck *et al.*, 1983). However, this enzyme was proposed to be involved only in assimilatory nitrate reduction. The bacterial symbionts of the clam *Lucinoma aequizonata* might use an anaerobic metabolic strategy (Cary *et al.*, 1989), and recently nitrate respiration has been demonstrated in the symbionts of *L. aequizonata* (U. Hentschel, pers. comm.) and *S. reidi* (Javor, pers. comm.). The sulfur metabolism of the whole clam under oxic and anoxic conditions in the presence of nitrate needs to be investigated.

This investigation was designed to address the following questions about sulfide and thiosulfate metabolism in the *S. reidi* symbiosis. (1) Is thiosulfate present in the mud around the clam and what causes thiosulfate to accumulate in clam blood? (2) Can the bacteria oxidize sulfide directly, and do bacteria and granules compete for sulfide? (3) Can the intact symbiosis use nitrate and nitrite as alternate electron acceptors?

Materials and Methods

Experimental animals

Specimens of *Solemya reidi* were collected at the Hyperion sewage outfall in Santa Monica Bay, California, at a depth of 100 to 140 m using a modified Van Veen grab. The animals were maintained in mud from their habitat in a flowing seawater aquarium. The aquarium was kept dark at 8°C to mimic natural conditions. All experimental incubations were done with animals maintained less than 30 days, with most animals maintained less than 14 days. Clam size varied from 32 to 42 mm. An effort was made to use similar size animals within experiments. Nitrate and nitrite respiration experiments (Figs. 1, 2) have all values normalized to a wet gill weight of 420 mg/clam. Blood values (Tables I, II, III) are not normalized to size or gill weight. The values represent the concentrations measured.

Whole clam sulfur use experiments (oxic and anoxic) were done with aquarium-maintained animals that had been pre-incubated for a minimum of 48 h in oxygenated seawater (dark at 8°C) without sulfur substrates. This preincubation allowed the animals to remove reduced sulfur compounds from their blood (see Results). All incubations were performed in a glass, water-jacketed incubation chamber with 500 ml of filtered seawater at 10°C. Whole clam nitrate and nitrite respiration experiments were done with aquarium-maintained animals immediately after removal from the mud. Incubations were done in ground-glass stoppered bottles with 100 ml filtered seawater at 10°C. All anoxic incubations were done in seawater sparged with argon for 10 min. The chambers were sealed

except for sampling, and argon was blown over the opening during sampling.

Sulfur compound sampling and analysis

Concentrations of sulfide, thiosulfate, sulfite, and glutathione were measured by HPLC using the monobromobimane (bimane) technique (Newton and Fahey, 1987; Vetter *et al.*, 1989). For porewater samples, the sediment surrounding individual animals was collected and immediately placed in 50-ml test tubes and centrifuged in a clinical centrifuge at approximately 2500 RPM. Aliquots of 100 μl were immediately derivatized with bimane. The reaction of bimane with the sample rapidly derivatized all reduced thiols and prevented further oxidation. Blood samples were collected by carefully cutting the membrane that connects the two valves and opening the animal. Seawater was wiped away, and the mantle was cut at the exhalent opening. Aliquots of 100 μl blood were immediately derivatized with bimane. Water samples from whole animal incubations were collected and 100 μl aliquots were immediately derivatized with bimane. Fluorescence of the bimane-derivatized samples was measured as previously described (Wilmot and Vetter, 1990).

Bacterial enrichments

Suspensions of bacterial symbionts were obtained by gently homogenizing the gills of aquarium-maintained clams in a glass homogenizer in filtered (0.45 μ*M*) seawater buffered with 10 m*M* MOPS (3-[N-morpholino]propanesulfonic acid), pH 7.5. The homogenate was centrifuged at low speed (53 × *g*) at 4°C for 5 min, pelleting the large cellular debris. Filtration of the suspension through 15-μm or 28-μm Nitex filters separated the bacteria (approximately 1 μm diameter) from the larger subcellular particles. The filtrate was centrifuged a second time at 1925 × *g* at 4°C for 5 min, which pelleted the bacteria and granules while leaving the mitochondria in the supernatant. The pellet was washed and resuspended in MOPS-buffered seawater. The Percoll gradient technique (Distel and Felbeck, 1988; Wilmot and Vetter, 1990) was not effective in separating the bacteria from other subcellular particles. Light microscopy was used to confirm that preparations did not contain nuclei and large cellular debris. Because mitochondria are too small to detect by casual observation, the bacterial preparation was tested for contamination by measuring ATP production. There was no external ATP produced, indicating that either no mitochondria were present or they could not function in seawater. Bacterial preparations had a final concentration of 36–38 mg wet gill tissue/ml seawater.

Bacterial enrichments were also made from clams that had been starved for 21 days. These animals were placed in oxygenated seawater containing no sulfur substrates or nitrate and kept at 8°C in the dark. The seawater was changed at least once a day. The bacterial suspensions were prepared exactly as described above.

Protein determination

Total protein of bacterial suspensions was measured by the method of Hartree (1972).

Nitrite determination

Seawater samples (1 ml) were collected and nitrite determined using a modification of the colorimetric method of Strickland and Parsons (1977). Due to interference by thiols, several modifications were necessary. Briefly, we had to solve the problem of interference by sulfide and thiosulfate. This was accomplished by precipitating the thiols.

Respiration measurements

All incubations were carried out in a Strathkelvin respiration chamber at 15°C. The respiration chamber was thermally jacketed and contained a stir bar that allowed homogeneous incubation at a constant temperature. Oxygen consumption was measured with a Clark-type oxygen electrode (Strathkelvin Instruments) modified to reduce interference by H_2S (O'Brien and Vetter, 1990).

Results

Sediment porewater and blood sulfur compounds

To determine which reduced sulfur compounds were available to the clams, we sampled the sediment porewater surrounding individual clams and in the blood of these clams. The major sulfur compound in sediment porewater was sulfide (Table I). Extremely low concentrations of thiosulfate were measured, but no sulfite or glutathione was present. Mean sulfide concentrations were highly variable between sediment samples at different sites (ranging from a low of 17 μ*M* to a high of 888 μ*M*). Variability within sediment samples was considerably less.

Although sulfide was the major reduced sulfur compound in the seawater surrounding the animals and thiosulfate concentrations were extremely low, clam blood contained high concentrations of thiosulfate (Table I). The blood of the animals also contained low concentrations of sulfide and sulfite (≤12 μ*M*). The cellular thiol compound, glutathione, remained relatively constant throughout sampling and was used as an indicator that hemolymph samples were not diluted by seawater. Nitrite was not present in either the sediment porewater or blood from freshly collected animals (data not shown).

To determine how fast thiosulfate and sulfide could be cleared from the blood of freshly collected animals from

Table I

Sulfur compounds from sediment porewater and blood of freshly collected Solemya reidi

Sample	n	Sulfur compounds (μM)			
		Sulfide	Thiosulfate	Sulfite	Glutathione
Pore water					
May 1987	6	385 ± 321	11 ± 19	ND	ND
June 1987	6	17 ± 5	1 ± 0	ND	ND
July 1990	6	38 ± 30	13 ± 2	0	0
September 1990 #1	4	20 ± 8	3 ± 1	0	0
September 1990 #2	6	888 ± 60	5 ± 4	0	0
Blood					
May 1987	6	4 ± 11	297 ± 120	ND	ND
June 1987	6	1 ± 0	111 ± 31	ND	ND
July 1990	7	11 ± 6	29 ± 17	<1	74 ± 27
September 1990 #1	4	8 ± 3	149 ± 35	9 ± 5	28 ± 13
September 1990 #2	5	12 ± 3	232 ± 89	9 ± 4	57 ± 21

June, 1987, data previously published (Vetter *et al.*, 1989). Values are mean ± standard deviation.
"ND" represents value not determined.

a single grab, 20 clams were placed in oxic seawater containing no reduced sulfur substrates. The removal of thiols from the blood of the clams is shown in Table II. An initial thiosulfate concentration of 274 ± 106 μM decreased to 17 ± 11 μM in 12 h and to zero in 24 h. Low concentrations of sulfide and sulfite were also cleared from the blood within 12 h while glutathione concentrations remained relatively constant.

Aerobic and anaerobic metabolism of sulfide and thiosulfate by whole animals

Sulfide and thiosulfate uptake and metabolism under oxic conditions were investigated using aquarium-maintained clams. Incubations with whole clams were for either 2 or 4 h. The animals were incubated in 500 ml filtered seawater at 10°C in the presence of either sulfide or thiosulfate.

Table II

Clearance of sulfur compounds from the blood of freshly collected Solemya reidi *during an incubation in sulfide-free, oxygenated seawater*

Sample	n	Sulfur compounds (μM)			
		Sulfide	Thiosulfate	Sulfite	Glutathione
T = 0 h	4	9 ± 1	274 ± 106	13 ± 1	19 ± 24
T = 6 h	4	17 ± 8	66 ± 44	27 ± 10	27 ± 29
T = 12 h	4	0 ± 0	17 ± 11	0 ± 0	9 ± 0
T = 24 h	4	0 ± 0	0 ± 0	2 ± 3	11 ± 2
T = 96 h	4	0 ± 0	0 ± 0	0 ± 0	18 ± 5

Values are mean ± standard deviation.

Whole clams incubated with sulfide (100 μM) showed very little sulfide or sulfite accumulation in the blood during the 2-h experiment (Table III). However, high concentrations of thiosulfate in the blood were observed. To examine the possibility that thiosulfate was actively transported by the clam, a thiosulfate ratio representing the ratio of the seawater thiosulfate concentration at the end of each incubation to the thiosulfate concentration in the blood at the end of each incubation was calculated. The thiosulfate ratio under oxic conditions was 1:300. Glutathione concentrations did not show any consistent pattern.

Similar experiments were performed by incubating clams with thiosulfate. Under these conditions, the clams did not concentrate thiosulfate in their blood above the concentration in the surrounding seawater during 2- and 4-h incubations with two thiosulfate concentrations (50 and 250 μM) (Table III). The thiosulfate ratio for either concentration was never greater than 1:1. Virtually no sulfide or sulfite was observed in the blood and glutathione again showed no consistent patterns.

We also investigated whole animal sulfide and thiosulfate metabolism under anoxic conditions. In these experiments, whole clams were incubated in seawater that was sparged with argon. Anoxic conditions were maintained throughout the incubations.

Anoxic incubations of clams with sulfide (100 μM) showed little sulfide or sulfite accumulation in the blood (Table III). However, unlike oxic incubations, blood thiosulfate concentrations did not increase substantially (24 μM as compared to 300 μM during anoxic incubations).

Thiosulfate was removed from the seawater and appeared in the blood of animals incubated under anoxic

Table III

Sulfur compounds from blood of Solemya reidi *after oxic and anoxic incubations with sulfide and thiosulfate. All incubations were in 500 ml seawater at 10°C*

Incubation conditions	n	Sulfur compounds (μM)				Thiosulfate ratio*
		Sulfide	Thiosulfate	Sulfite	Glutathione	
Sulfide						
Oxic—2 h						
Control	4	0 ± 0	18 ± 1	0 ± 0	13 ± 10	
100 μM	6	1 ± 1	300 ± 217	1 ± 1	15 ± 5	1:300
Anoxic—2 h						
Control	4	8 ± 3	8 ± 5	4 ± 3	9 ± 7	
100 μM	6	6 ± 2	24 ± 23	7 ± 1	33 ± 19	1:24
Thiosulfate						
Oxic—2 h						
Control	3	2 ± 2	5 ± 3	4 ± 1	9 ± 10	
250 μM	3	4 ± 1	136 ± 0	7 ± 3	38 ± 9	1:0.8
Oxic—4 h						
250 μM	3	3 ± 2	152 ± 4	6 ± 4	38 ± 2	1:0.9
Oxic—2 h						
Control	4	2 ± 2	22 ± 13	12 ± 4	28 ± 20	
50 μM	4	3 ± 5	35 ± 11	9 ± 9	16 ± 4	1:0.8
250 μM	4	0 ± 0	157 ± 17	3 ± 2	11 ± 1	1:0.8
Anoxic—2 h						
Control	3	2 ± 2	5 ± 3	4 ± 1	9 ± 10	
250 μM with 5 mM nitrate	3	6 ± 2	158 ± 63	4 ± 1	14 ± 12	1:0.7
250 μM w/out 5 mM nitrate	3	3 ± 2	116 ± 33	4 ± 2	25 ± 19	1:0.5

Sulfur compound values are mean ± standard deviation.

* The thiosulfate ratio represents the ratio of the seawater concentration at the end of each incubation to the concentration in the blood. The concentration of thiosulfate in the seawater at the end of the sulfide incubations was below the limits of detection, thus a value of 1 μM was assigned. Controls represent animals after oxic or anoxic incubations without a reduced sulfur substrate (either sulfide or thiosulfate).

conditions both with and without nitrate (Table III). The pattern of blood thiols under anoxic conditions was similar to that for oxic conditions. Thiosulfate was not concentrated in the blood above the concentrations of the surrounding seawater under any of the incubation conditions.

Sulfide and thiosulfate stimulated nitrate/nitrite respiration

The intact symbiosis exhibited nitrate respiration under oxic and anoxic conditions in the presence of sulfide and thiosulfate (Fig. 1a, b). In the presence of 5 mM nitrate, nitrite accumulated in filtered seawater during 3-h oxic incubations with whole clams in the presence of 150 μM sulfide and 250 μM thiosulfate (Fig. 1a). Two different controls were run to confirm that nitrate respiration was being carried out by the symbiotic bacteria. First, 5 mM nitrate was added to filtered seawater containing 150 μM sulfide without clams. No nitrite accumulated in the seawater (not shown). Second, 5 mM nitrate was added to filtered seawater containing clams and no sulfide (Fig. 1a). Blood nitrite concentrations at the end of the 3-h incubations were 158 ± 80 μM (n = 4) and 52 ± 16 μM (n = 4) for sulfide- and thiosulfate-incubated clams, respectively.

Anoxic incubations were also performed. Whole clams had the potential for nitrate respiration under anoxic conditions (Fig. 1b). Three different controls were run. First, 5 mM nitrate was added to filtered, deoxygenated seawater containing 150 μM sulfide without clams. No nitrite accumulated in the seawater (Fig. 1b). Second, no nitrate was added to filtered, deoxygenated seawater containing 250 μM thiosulfate with clams. No nitrite accumulated in the seawater (data not shown). Finally, 5 mM nitrate was added to filtered, deoxygenated seawater containing 150 μM sulfide with clams that had their gills removed. Very little nitrite (2.0 ± 0.2 μM) accumulated in the seawater (Fig. 1b). Blood nitrite concentrations at the end of 3-h incubations were 214 ± 103 μM (n = 4) and 189 ± 80 μM (n = 4) for sulfide- and thiosulfate-incubated clams, respectively.

Five mM nitrate concentrations are not environmentally realistic. Because nitrite accumulation (*versus* nitrate disappearance) was measured and nitrite only accumulated in the presence of excess nitrate, it was essential that excess nitrate be available to the clams during the entire

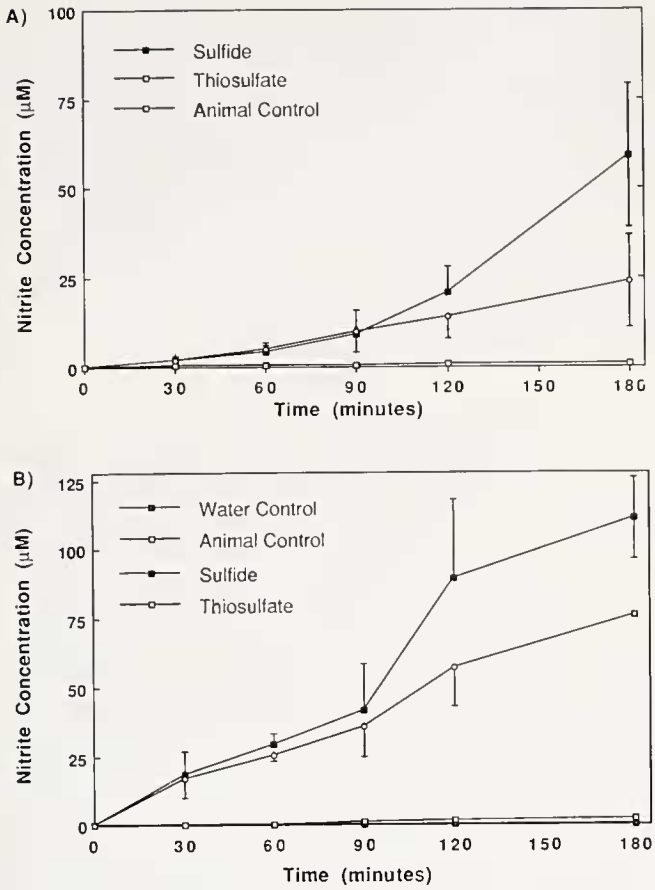

Figure 1. Nitrate respiration of whole *Solemya reidi* during 3-h incubations in oxic and anoxic buffered seawater at 10°C. (A) Nitrite accumulation in oxic seawater during incubations containing 5 m*M* nitrate and either 150 μM sulfide (mean ± standard deviation of 4 incubations) or 250 μM thiosulfate (mean ± standard deviation of 4 incubations). The animal control represents seawater containing 5 m*M* nitrate, animals, and no sulfide. (B) Nitrite accumulation in anoxic seawater during incubations containing 5 m*M* nitrate and either 150 μM sulfide (mean ± standard deviation of 4 incubations) or 250 μM thiosulfate (mean ± standard deviation of 4 incubations). The water control represents seawater containing 5 m*M* nitrate, 150 μM sulfide, and no animals. The animal control represents seawater containing 5 m*M* nitrate, 150 μM sulfide, and 4 animals with their gills removed. Clam size ranged from 32 to 42 mm and all values are normalized to a wet gill weight of 420 mg/clam.

3-h incubation. However, whole animal nitrate respiration (nitrite accumulation) rates similar to those presented above were found when clams were incubated in 500 μM and 50 μM nitrate for short incubations (only single experiment—data not shown). Similar results have also been found for bacterial preparations (Javor, pers. comm.).

The intact symbiosis showed nitrite respiration under anoxic conditions in the presence of sulfide but not thiosulfate (Fig. 2). In the presence of 150 μM sulfide, 100 μM nitrite was removed from filtered seawater by whole clams incubated anoxically. An initial nitrite concentration of 101.5 ± 2.8 μM decreased to 69.9 ± 6.0 μM during a 3-h incubation. Two different controls were run to confirm that nitrite respiration, like nitrate respiration, was being carried out by the symbiotic bacteria. First, 100 μM nitrite was added to filtered, deoxygenated seawater containing 150 μM sulfide and no clams. The seawater nitrite concentration did not decrease (Fig. 2). Second, nitrite was added to filtered, deoxygenated seawater containing no reduced sulfur compound and three clams. Again, the seawater nitrite concentration did not decrease (data not shown). Blood nitrite concentrations at the end of 3-h incubations were 5.3 ± 2.5 μM and 17.3 ± 4.6 μM for sulfide and thiosulfate incubations, respectively.

Sulfide-stimulated oxygen consumption by bacterial symbionts

The whole animal experiments could not directly address the question of whether the bacteria could carry out respiratory sulfide oxidation. To determine if the symbionts could respire sulfide aerobically, we measured azide-sensitive (respiratory) and azide-insensitive (non-respiratory) oxygen consumption of bacterial suspensions in the presence of different sulfide concentrations. The bacterial suspensions contained granules because they could not be separated from the bacteria. The suspensions oxidized a wide range of sulfide concentrations with maximal respiration rates at 25 μM sulfide (Fig. 3a). A pairwise comparison of values at 25 μM and all higher concentrations using the Mann-Whitney U test (Zar, 1984) found no significant difference between 25 μM and any higher values ($P > 0.05$). However, the number of repli-

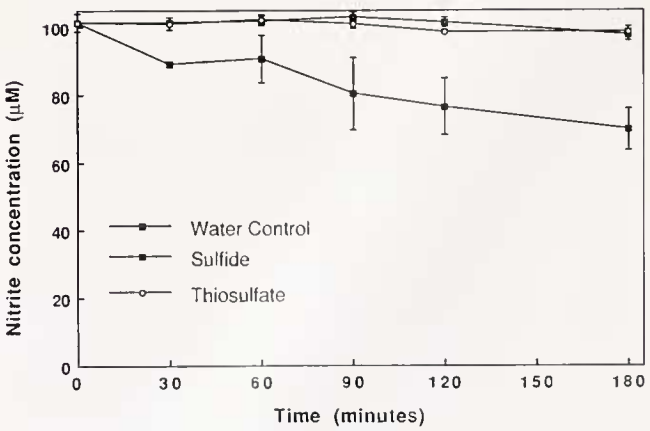

Figure 2. Nitrite respiration of whole *Solemya reidi* during 3-h incubations in anoxic buffered seawater at 10°C. Nitrite disappearance in seawater during incubations containing either 200 μM sulfide (mean ± standard deviation of 4 incubations) or 200 μM thiosulfate (mean ± standard deviation of 4 incubations). The water control represents seawater containing 100 μM nitrite, 150 μM sulfide, and no clams. Clam size ranged from 32 to 42 mm and all values are normalized to a wet gill weight of 420 mg/clam.

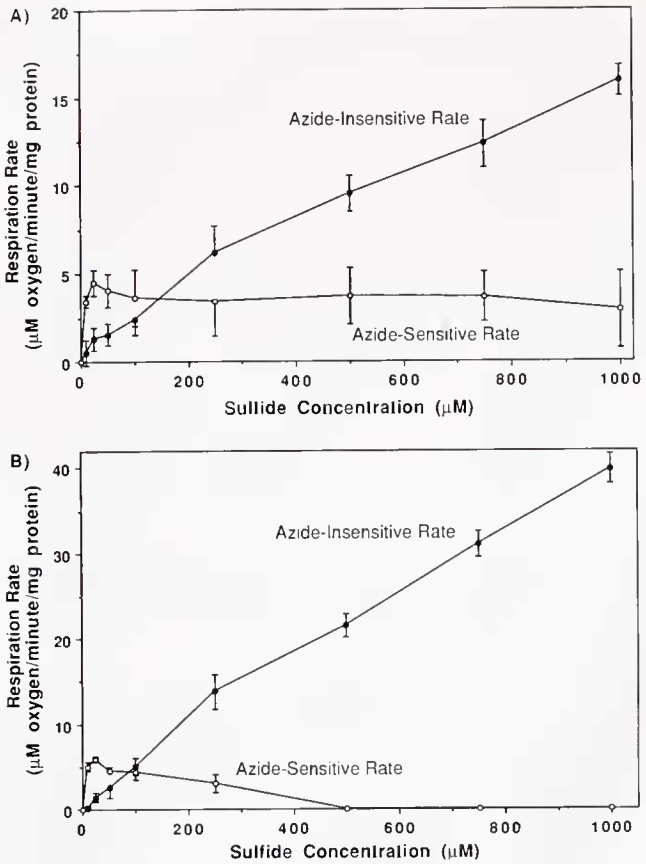

Figure 3. Sulfide-stimulated oxygen consumption in buffered seawater at 15°C by bacterial enrichments from (A) the gills of mud-maintained *Solemya reidi* or (B) the gills of clams maintained in oxygenated seawater that contained no reduced sulfur compounds or nitrate for 21 days. Data points represent means and standard deviations from three experiments (n = 3). See Materials and Methods for clam size and bacterial density.

cates (n = 3) is not sufficient for a robust statistical determination. Thus, aerobic respiration was apparently not inhibited by sulfide concentrations up to 1 m*M*. However, the azide-insensitive oxygen consumption rate increased over the range of sulfide concentrations tested. The maximal rate for azide-insensitive consumption was above 1 m*M*. The data in Figure 3a are the combination of the means from multiple measurements in three different experiments (each experiment was done with one bacterial preparation consisting of many clams).

Similar respiration experiments were conducted on bacterial suspensions from clams that were maintained in oxygenated seawater without an external reduced sulfur source or nitrate for three weeks. The gills of these animals were dark (no yellow sulfur globules). The total number of bacteria or a bacteria to granule ratio was not determined. However, it has been observed that starved clams have more granules than healthy clams (pers. obs.). The bacterial suspensions from these gills showed a different pattern for azide-sensitive and insensitive oxidation rates (Fig. 3b). Azide-sensitive aerobic respiration again had a maximum rate at 25 μM, but appeared to be inhibited at higher concentrations with complete inhibition at 500 μM and greater sulfide concentrations. The azide-insensitive rate increased with increasing sulfide concentrations and was more than double that found in the bacterial enrichments from animals freshly removed from mud based on total protein of the suspensions. The data in Figure 3b are the combination of three different experiments.

Discussion

Analysis of sediment porewaters showed that sulfide was typically the only reduced sulfur compound available to *S. reidi* and that concentrations ranged from below 20 μM to nearly 1 m*M*. Because the clams actively pump water from above the sediment through their burrows, sulfide concentrations in the water that is in contact with the gills may be significantly lower. Porewater thiosulfate was present in extremely low concentrations, and the clams were unable to accumulate thiosulfate in the blood above the external concentration. Thus, it appears that porewater thiosulfate is of little importance as an energy source in the intact symbiosis.

Similar to Anderson *et al.* (1987), we observed high concentrations of thiosulfate in the blood of field caught animals and animals experimentally incubated in the presence of sulfide. Our data show that thiosulfate present in the blood of freshly collected clams results from host oxidation of sulfide and not from uptake of porewater thiosulfate. Because thiosulfate accumulates in the blood during oxic incubations with sulfide but not during anoxic incubations, molecular oxygen must be required for sulfide oxidation to thiosulfate. It is most likely that aerobic respiration of sulfide by mitochondria is responsible for thiosulfate production (O'Brien and Vetter, 1990). Thiosulfate is cleared from the blood by the bacteria within bacteriocytes. However, the relative importance of this energy source *versus* direct uptake of sulfide across the gill is not known.

Two clearly different types of sulfide oxidation occurred in our bacterial enrichments. The first, true respiration, represents electron transport chain (ETS)-linked bacterial sulfide oxidation and it is completely azide-sensitive. The ETS-linked type showed a high capacity to oxidize sulfide at low concentrations (maximal rate by 25 μM). The second, which is azide-insensitive, represents non-enzymatic oxidation by ferric iron catalysis (hematin) in granules (Powell and Arp, 1989) and autocatalysis by sulfur (Chen and Morris, 1972).

Non-enzymatic, heat-stable catalysis of sulfide oxidation has been observed in a variety of animal tissues (re-

viewed by Beauchamp *et al.*, 1984) and specifically in a thiotrophic symbiosis (Wilmot and Vetter, 1990). It has been shown that ferric iron-containing compounds such as hematin and ferritin are responsible for the non-enzymatic catalysis in mammals (Sörbo, 1958; Baxter and van Reen, 1958; Baxter *et al.*, 1958). The benzyl viologen (BV) assay used to determine which components of the *S. reidi* symbiosis carried out sulfide oxidation (Powell and Somero, 1985) measures non-enzymatic ferric iron catalysis in the presence of high (1–5 m*M*) sulfide (Powell and Arp, 1989). It does not measure ETS-linked activity because high sulfide concentrations inhibit the oxidation. Thus, it is not surprising that early studies using the BV assay concluded that the bacteria and mitochondria did not oxidize sulfide (Powell and Somero, 1985).

Although we have shown that the bacteria are capable of sulfide oxidation, it is not clear whether the bacteria encounter sulfide or if it is first oxidized by mitochondria or electron-dense granules. Several lines of evidence suggest that the bacteria normally oxidize sulfide as well as thiosulfate *in vivo*. First, the bacteria are oriented close to the outside and are separated from the seawater by a thin epithelial cell that contains few mitochondria or electron-dense granules (Felbeck, 1983; Gustafson and Reid, 1988). Because the bacteria are not packed close to the blood space, which contains the mitochondrial product of sulfide oxidation, it does not seem likely that thiosulfate is the only sulfur substrate available to the bacteria. Second, isolated gill ctenidia can oxidize sulfide without a host blood supply (Vetter, 1990). The ctenidia produce elemental sulfur and protein, which seems to indicate that sulfide and oxygen are taken directly across the gill (Vetter, 1990). Third, the bacteriocytes of *S. reidi* and *S. velum* contain intracellular hemoglobin that can bind sulfide as ferric hemoglobin sulfide (Doeller *et al.*, 1988; Krause and Wittenberg, 1990). Fiber-optic spectroscopy of intact gills indicated that ferric hemoglobin sulfide was present in the bacteriocyte region of the gill.

The data presented above do not provide direct proof that the bacteria within a bacteriocyte oxidize sulfide. Javor (pers. comm.) has recently observed that bacterial suspensions from *S. reidi* respire nitrate under oxic and anoxic conditions in the presence of sulfide and thiosulfate. Similarly, in this study, the symbionts respired nitrate that entered the bacteriocyte, either from the blood, or directly from seawater in the presence of sulfide and thiosulfate. The product of the anaerobic respiration, nitrite, was excreted from the bacteriocyte in the presence of excess nitrate and accumulated in the blood and seawater. In marine denitrifying bacteria, nitrate is often reduced only to nitrite (Goering and Cline, 1970) and the nitrite accumulates outside the cells (Payne and Riley, 1969). If nitrate concentrations become low or are exhausted, the nitrite can be taken back up by the bacteria and further reduced.

In the absence of nitrate, the intact symbiosis respired nitrite in the presence of sulfide only. This has also been observed for bacterial suspensions from *S. reidi* (Javor, pers. comm.). The nitrite, like the nitrate, either enters the bacteriocyte from the blood or directly from seawater. More importantly, these data provide the best available evidence that the bacteria within bacteriocytes oxidize sulfide. These results suggest that sulfide oxidation is coupled to complete denitrification, but that thiosulfate oxidation is coupled to the first step only. Anaerobic sulfide oxidation by the bacteria may be an important detoxification mechanism when the clam is depleted of oxygen. In addition, because aerobic nitrate respiration has been described for several bacteria (Robertson and Kuenen, 1984; Lloyd *et al.*, 1987) denitrification may occur when nitrate (and sulfide) is present and oxygen concentrations are low.

Although the symbionts are capable of denitrification, and the bacteria have access to nitrate and nitrite when each is present in seawater, we do not know the concentrations that are available to the animals in the natural environment. The water flow across the sludge field is strong and the oxygen concentrations and water chemistry are similar to the surrounding areas (approximately 100–175 μM O_2) (B. Thompson, pers. comm.). At neighboring hydrographic stations in the Santa Monica Bay area, the ammonia concentrations range from 0.5 to 2.0 μM, while sediment porewaters typically are 100–1000-fold higher (Eppley, 1986). Nitrifying bacteria are active in these waters (Ward *et al.*, 1982) and nitrate values at a depth equal to the sludge field are $\geq$20 μM (Williams, 1986). Nitrate is not present below the top 2 cm of sediment at Whites Point outfall near the Hyperion outfall (J. Gieskes, pers. comm.). It can be assumed that as the clams pump water from above the sediment through their burrows, they are probably exposed to oxygen and nitrate simultaneously, but the ratio of oxygen to nitrate is unknown.

When the data presented here are integrated with previous studies (Felbeck, 1983; Powell and Somero, 1985, 1986; Fisher and Childress, 1986; Anderson *et al.*, 1987, 1990; Doeller *et al.*, 1988; Krause and Wittenberg, 1990; O'Brien and Vetter, 1990), a more complete picture emerges of how the symbiosis may be functioning under different environmental conditions. Maximum net autotrophy occurs in the presence of external sulfide concentrations of 100 μM (Anderson *et al.*, 1987). Because seawater is near pH 8.1 at 100 μM total sulfide, approximately 3 μM sulfide as H_2S diffuses into animal cells (Millero, 1986). Assuming an internal pH near pH 7.5, the maximal internal free sulfide (H_2S) concentration is approximately 20 μM, which is very close to the aerobic sulfide-oxidizing maximum for both isolated mitochon-

dria (O'Brien and Vetter, 1990) and bacteria. Aerobic metabolism is maintained by animal tissues in the presence of external sulfide concentrations up to 100 μM. At higher concentrations, the onset of anaerobic pathways is evident (Anderson *et al.*, 1990).

Sulfide oxidation can occur by at least two processes: sulfide respiration by mitochondria and bacteria, and non-enzymatic catalysis by metal containing granules, and autocatalysis by sulfur. The mitochondria oxidize sulfide only to thiosulfate which accumulates in the clam's blood (O'Brien and Vetter, 1990). The oxidation is oxygen-dependent and can yield ATP (Powell and Somero, 1986). Presumably, the bacteria oxidize sulfide to elemental sulfur and polysulfides and ultimately to sulfate. Bacterial sulfide respiration can occur aerobically when oxygen is available or possibly anaerobically when nitrate (or nitrite) is available as an alternate electron acceptor. The importance of nitrate (nitrite) respiration is unknown. In addition, the thiosulfate produced by the mitochondria can be further oxidized by the bacteria. The importance of the non-enzymatic oxidation of sulfide is unknown.

We have shown the metabolic potential for the use of nitrate and nitrite as alternate electron acceptors in the intact symbiosis. Future studies must determine the environmental concentrations of nitrate, nitrite, and oxygen and the relevance of anaerobic respiration to the symbiosis. If oxidized nitrogen compounds are present, they are likely in the oxygen-containing water drawn into the burrow by the clams. Thus, conditions that limit oxygen availability would also limit nitrate. A reasonable hypothesis may be that a switch to anaerobic nitrogen respiration by the symbionts is a tactic by the symbiosis to save oxygen for host metabolism during times of low oxygen.

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